

# Detection of mRNA by Reverse Transcription PCR as an Indicator of Viability in *Phytophthora ramorum*<sup>1</sup>

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## Abstract

Real-Time PCR technologies offer increasing opportunities to detect and study phytopathogenic fungi. They combine the sensitivity of conventional PCR with the generation of a specific fluorescent signal providing both real-time analysis of the reaction kinetics and quantification of specific DNA targets. Before the development of Real-Time PCR and the opportunity to provide quantitative data, the risk of false-positive PCR results due to detection of dead cells was considered only a minor setback. This, therefore, has led to a renewed interest in the risk of false-positive PCR results. In order to deal with this potential problem we developed a new reverse transcript (RT) PCR assay based on the use of mRNA as a viability marker, on the basis of its rapid degradation compared to DNA. We developed new primers, specific for *P. ramorum*, designed in the cytochrome oxidase gene encoding subunits I (COXI). To evaluate the specificity of the method, four isolates of *P. ramorum* and 11 different *Phytophthora* species were tested. One hundred symptomatic bay leaves from three different sites in California were collected in three different seasons of the year. Samples were plated on PARP selective media for *Phytophthora* and tested with the new RT-PCR method and compared with a TaqMan and SybrGreen Real-Time PCR assay after DNA extraction. Results showed that after seven days RNA of freeze-dried killed *P. ramorum* was undetectable while DNA gave a positive signal. Furthermore, data from the new assay were more correlated to the results obtained after isolation on selective media whereas DNA-based results showed more positive samples. This indicates that by using the new RT-PCR method, the risk of false-positive PCR results due to detection of dead cells can be minimized.

Key words: *Phytophthora ramorum*, RT PCR, dead cells, false positives, diagnosis.

## Introduction

*Phytophthora ramorum* (Werres, de Cock and Man in 't Veld), causal agent of sudden oak death (SOD), is one of the best-known examples of an exotic pathogen which has killed thousands of oak trees along the U.S. west coast, causing a dramatic decline of local forests in terms of structure and biodiversity.

The development of accurate and rapid diagnostic methods for *P. ramorum* is crucial for providing the tools useful for detecting the pathogen and determining its potential for establishing itself in new regions.

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Real-Time PCR technologies offer increasing opportunities to detect and study phytopathogenic fungi. They combine the sensitivity of conventional PCR with the generation of a specific fluorescent signal providing both real-time analysis of the reaction kinetics and quantification of specific DNA targets (Schmittgen 2001). One disadvantage of DNA-based methods is that they do not distinguish between living and dead organisms, which limits their use for monitoring purposes. In order to deal with risks of false-positive PCR results, many researchers have investigated the use of mRNA as a viability marker, on the basis of its rapid degradation compared to DNA (Alifano and others 1994).

The principal objective of this research programme is to define scientific parameters to discriminate dead cells from living cells of *P. ramorum* present in plant material and develop a reliable and sensitive molecular method to assess the risk of false positive in the detection of *P. ramorum* from environmental samples.

Real-Time PCR was used to detect the expression of the cytochrome oxidase 1 gene in order to examine the relationship between viability and presence of mRNA, since any relationship between mRNA and viability may depend on the method used to inactivate cells or the type of mRNA sought. We exposed the cells to three different stress treatments (heat, lyophilization and ethanol) and assayed mRNA from the cytochrome oxidase 1 gene. Furthermore, the method was validated testing symptomatic bay leaves collected in different seasons from three sites in California. Results obtained with RT-PCR were compared with those obtained by traditional isolation on PARP selective medium and Nested TaqMan and SYBR Green PCR.

## Materials and Methods

### *Sampling and Isolation*

The survey was carried out in California and the chosen sites were: 1) China Camp State Park, which lies along San Pablo Bay near San Rafael; 2) Briones Regional Park on the eastern side of San Francisco Bay and 3) Samuel P. Taylor State Park located in Marin County north of San Francisco. Sites were selected to encompass a range of habitat types and species compositions found within *P. ramorum* infested forests.

Between October 2005 and July 2006, each site was inspected for the presence of SOD. China Camp State Park was sampled three times (October, April, July), whereas Samuel P. Taylor and Briones Parks were sampled only in July. At each inspection symptomatic leaves from 20 California bay laurel trees were randomly chosen, collected and taken to the laboratory (total of 100 leaves). Leaves were superficially wiped with 70 percent EtOH and then divided in three homogeneous sectors containing approximately the same amount of lesions and healthy tissues. Each piece, randomly chosen, was assigned to one the different treatments (direct plating on PARP selective medium, DNA or RNA extraction) in order to compare different diagnostic methods.

Asymptomatic California bay laurel leaves, collected from the University of California (UC) Berkeley campus, were used as negative control.

### **Inactivation Treatments for RT-PCR**

In order to validate the new diagnostic approach mRNA was recovered from pure cultures of *P. ramorum* (isolates Pr1, Pr52, Pr72 and Pr102) that had been subjected to treatments designed to impact cell viability.

Isolates Pr1, Pr52, Pr72 and Pr 102 were cultured on pea broth for 7 to 10 days and then the mycelium was harvested, washed with sterile water and then treated with 70 percent EtOH for 1 hour, heat-treated at 60°C for 1 hour or freeze-dried (lyophilized) overnight in order to inactivate the cultures. Treated isolates were plated on V8A in order to monitor the presence of viable *P. ramorum* cells. All the treated mycelia were left at room temperature for 0, 1, 2, 5, 7, 9 and 12 days and then kept at -80°C before proceeding with RNA and DNA extraction.

### **Nucleic Acids Extraction**

Mycelium and plant material was lyophilized before extractions. DNA from approximately 50 mg of mycelium was extracted using a PureGene DNA Extraction Kit (Gentra) according to the manufacturer's instructions. DNA from approximately 50 mg of lyophilized bay leave tissue was extracted according Hayden and others(2004). RNA was extracted from approximately 50 mg of mycelium or approximately 50 mg of symptomatic bay leaves using RNeasy® Plant Mini Kit (Qiagen) following the manufacturer's protocol. RNA was purified from DNA contamination using RNase-Free DNase Set (Qiagen).

### **Primer Sequence Design and Specificity**

The COXI sequence of *P. ramorum* was utilised to design specific primers to amplify DNA fragments from this particular species. Sequences of *P. ramorum* and related species were aligned using the ClustalW software (EMBL, European Bioinformatics Institute) and screened for base pair differences and then best primer sets were designed using Primer3 software. Specificity of ACPrmF and ACPrmR primer set was preliminarily assessed by means of Basic Local Alignment Search Tool (BLAST) analyses to explore all of the available sequence DNA databases and exclude the presence of similar sequences in other microorganisms. Furthermore, the specificity of these primers was assessed using genomic DNA from other species of *Phytophthora* (table 1).

### **Two-Step RT-PCR**

The presence of target COXI RNA was analyzed by two step RT-PCR using QuantiTect® Reverse Transcription Kit (Qiagen) following the manufacturer's procedure. Each purified RNA sample was briefly incubated in gDNA Wipeout Buffer (Qiagen) at 42°C for two minutes to efficiently remove any possible contaminating genomic DNA.

cDNA was then amplified using ACPrmF/ACPrmR primer set. PCR was performed in a total volume of 25µl containing 6.26µl of undiluted cDNA combined with 12.5µl of 2x iQTM SYBR® Green Supermix (Bio-Rad) containing 0.5 mM of each primer. Real-Time amplification was carried out in an iCycler IQ Real-Time

thermocycler (Bio-Rad) using the following conditions: 1 cycle at 50°C for 10 minutes, 1 cycle at 95°C for 3 minutes, 40 cycles at 95°C for 15 seconds, and 58°C for 1 minute. Ramp rate was 3.3°C/s heating and 2.0°C/s cooling.

For each PCR run with SYBR Green detection, a melting curve analysis was performed to guarantee the specificity in each reaction tube (absence of primer dimers and other non-specific products).

**Table 1—*Phytophthora* species used to determine specificity of the *Phytophthora ramorum* specific primers**

| Species                    | Local isolate no. | Host                            | Origin     | Amplification       |
|----------------------------|-------------------|---------------------------------|------------|---------------------|
| <i>P. cambivora</i> *      | MP14              | <i>Quercus agrifolia</i>        | California | Not cross-amplified |
| <i>P. cambivora</i> *      | MP22              | Almond                          | California | Not cross-amplified |
| <i>P. cambivora</i> *      | NY217             | Apple                           | New York   | Not cross-amplified |
| <i>P. cambivora</i> *      | NY249             | Apple                           | Oregon     | Not cross-amplified |
| <i>P. citricola</i> *      | MP18              |                                 | California | Not cross-amplified |
| <i>P. cryptogea</i> *      | MP11              | <i>Lycopersicon esculentum</i>  |            | Not cross-amplified |
| <i>P. drechsleri</i> *     |                   |                                 |            | Not cross-amplified |
| <i>P. hibernalis</i>       |                   |                                 |            | Not cross-amplified |
| <i>P. nemorosa</i> *       | MP16              |                                 | California | Amplified >1 pg     |
| <i>P. lateralis</i> **     | PL27              | <i>Chamaecyparis lawsoniana</i> | California | Not cross-amplified |
| <i>P. megasperma</i> *     | MP20              | <i>Glycine max</i>              | Wisconsin  | Amplified >1 pg     |
| <i>P. palmivora</i> *      | MP8               | <i>Theobroma cacao</i>          |            | Amplified >1 pg     |
| <i>P. pseudosyringae</i> * | P40               | <i>Quercus agrifolia</i>        | California | Amplified >1 pg     |
| <i>P. syringae</i> *       | MP15              | <i>Rhododendron</i> spp.        | California | Not cross-amplified |
| <i>P. ramorum</i> **       | Pr1               | <i>Quercus agrifolia</i>        | California | Amplified           |
| <i>P. ramorum</i> **       | Pr52              | <i>Rhododendron</i> sp.         | California | Amplified           |
| <i>P. ramorum</i> **       | Pr72              | <i>Rhododendron</i> sp.         | California | Amplified           |
| <i>P. ramorum</i> *        | Pr102             | <i>Quercus agrifolia</i>        | California | Amplified           |

\* isolates used in Hayden and others (2004). \*\* Isolates used in Hayden and others (2006).

### **Nested SYBR Green Real-Time PCR**

The first and second round of PCR amplification were performed using primer sets Phyto1/Phyto4 and Phyto2/Phyto3, respectively following the procedure described by Hayden and others (2004).

### **Nested TaqMan Real-Time PCR**

TaqMan reaction was performed using primer set Phyto1/Phyto4 (1<sup>st</sup> round) and primers Pram5/Pram6 and probe Pram7 (2<sup>nd</sup> round) following the same conditions previously described by Hayden and others (2006).

## **Results and Discussion**

### **Detection of mRNA From Killed Cells**

Of the three different inactivation treatments chosen, only lyophilized, *P. ramorum* did not grow when plated after the treatment. Heat-treated mycelium of *P. ramorum* remained active, whereas all EtOH-treated samples were inactivated after three weeks incubation with the exception of isolate Pr52 (two of four plates) that was still active after three months (showing sparse mycelium).

mRNA from the COXI gene was detected by RT-PCR in all the samples immediately after treatments. However, during subsequent incubation at room temperature, mRNA of lyophilize-treated samples became undetectable after seven days, whereas all other samples gave a positive RT-PCR amplification (table 2). Samples used as negative control never showed a positive amplification or any growth on plates.

**Table 2—mRNA detected by RT-PCR after incubation of treated *P. ramorum* mycelium at room temperature**

| Treatment                  | Target | Incubation at room temperature * |       |        |        |        |        |         |
|----------------------------|--------|----------------------------------|-------|--------|--------|--------|--------|---------|
|                            |        | 0                                | 1 day | 2 days | 5 days | 7 days | 9 days | 12 days |
| Untreated                  | COXI   | Y                                | Y     | Y      | Y      | Y      | Y      | Y       |
| 60° C for 1 hour           | COXI   | Y                                | Y     | Y      | Y      | Y      | Y      | Y       |
| EtOH 70 percent for 1 hour | COXI   | Y                                | Y     | Y      | Y      | Y      | Y      | Y       |
| Lyophilized                | COXI   | Y                                | Y     | Y      | Y      | N      | N      | N       |

\* Y, positive RT-PCR amplification; N, negative RT-PCR amplification.

### Comparison of Traditional and Molecular Diagnostic Methods

One hundred bay leaves samples, showing SOD symptoms, were assayed for the presence of *P. ramorum* with four different diagnostic methods: isolation on PARP selective medium, COXI RT PCR, Nested TaqMan PCR, and SYBR Green PCR. The July survey samples that were collected in all three sites had the most xeric conditions and therefore the detection of *P. ramorum* was low with all four assays. Isolation with selective PARP medium gave three positive (15 percent) out of 20 samples, whereas molecular RNA and DNA assays gave 25 percent and 30 percent positives, respectively. All the samples collected in China Camp State Park were positive when tested with Nested TaqMan and SYBR Green PCR (100 percent), whereas RT-PCR and PARP isolation gave 45 percent and 35 percent of positives, respectively. Samples collected in Samuel P. Taylor State Park, which is considered to be the most mesic site, were 5 percent positive when plated on PARP, 50 percent when assayed with RT-PCR and 75 percent when assayed with TaqMan or SYBR Green assays (fig.1).

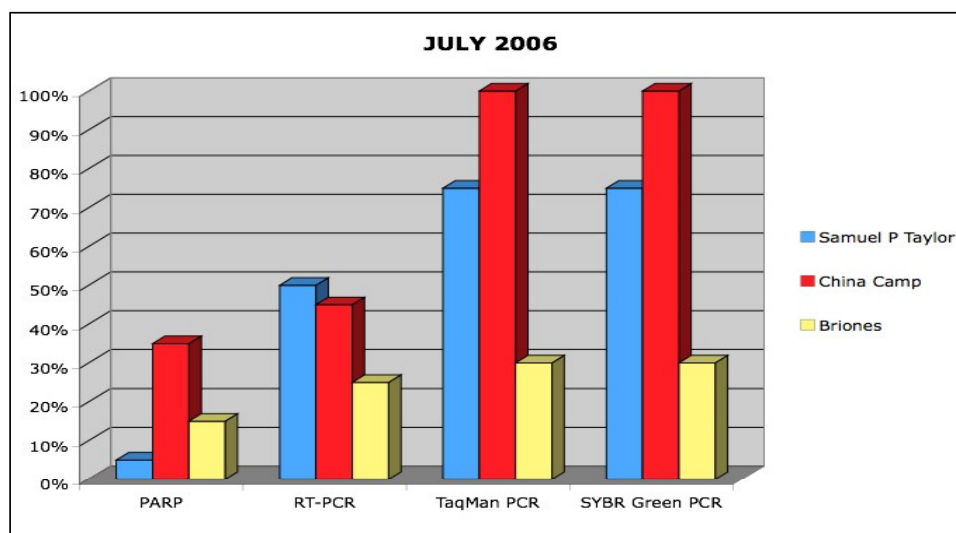


Figure 1—Comparison of four different methods to detect *P. ramorum* from symptomatic California bay leaves. Samples were collected in July 2006 from three different sites: Samuel P Taylor State Park, China Camp State Park and Briones Regional Park.

Frequency of detection of *P. ramorum* from naturally infected California bay leaves differed significantly across sites and between methods of detection. Nevertheless, both Nested DNA assays, detection via SYBR Green and DNA detection via a TaqMan probe, were equally sensitive with 78 percent and 77 percent of positive samples respectively (fig. 2).

Traditional isolation with PARP selective medium was a less sensitive and reliable assay for the detection of *P. ramorum* compared to the molecular methods. In fact, only 38 percent out of all tested samples resulted positive using this approach, which required more time and effort for the isolation and the identification.

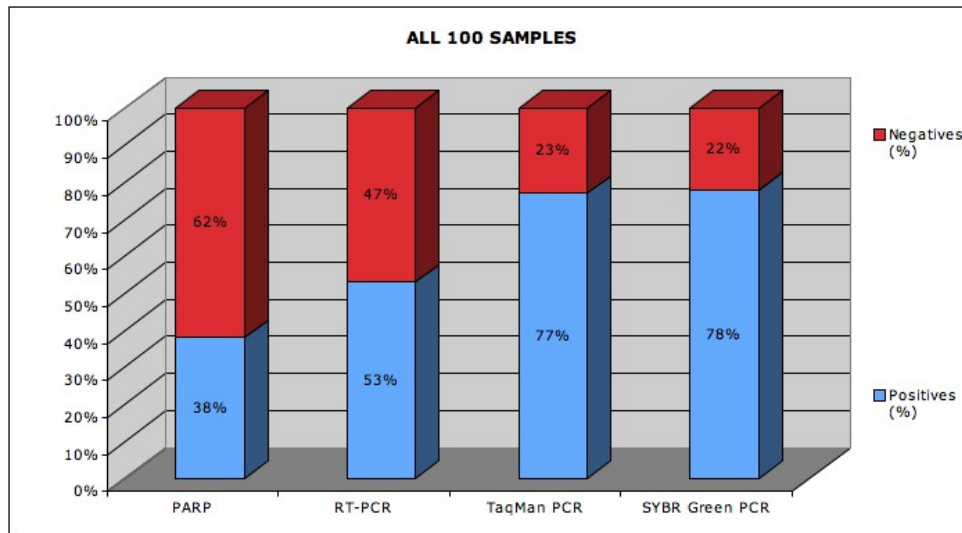


Figure 2—Comparison of four different methods to detect *P. ramorum* from symptomatic California bay leaves. Samples were collected from 100 different trees in China Camp State Park in October 2005, April 2005 and July 2006; and in Briones Regional Park and in Samuel P. Taylor State Park in July 2006.

Detection of *P. ramorum* by RT-PCR was significantly more sensitive compared to traditional isolation on PARP selective medium, but differed from DNA based detection assays with 53 percent positive out of 100 samples tested. Furthermore, all samples that were positive on PARP were positive with all three molecular methods and all RT-PCR positive samples were positive with DNA based diagnostic methods.

It should be pointed out that *P. ramorum* remained active after 1 hour at 60°C and after one hour in 70 percent EtOH. The only treatment able to devitalize *P. ramorum* was lyophilization. Results showed a good correlation between the presence of mRNA and the viability of *P. ramorum* when RNA was extracted from freeze-dried mycelium. The detection of mRNA therefore indicates either that a cell is alive or has died fairly recently. We have established that mRNA can persist for at least seven days in lyophilized mycelium of *P. ramorum*, but further analysis is required to characterize the decay rates of mRNA in dead cells in a range of conditions before the limitations of the method are fully defined. This study has demonstrated that mRNA is a promising candidate as an indicator of viability *P. ramorum*, although further evaluation with additional *Phytophthora* spp. is needed to confirm species-specificity of the diagnostic primers.

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